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Protective effects of certain lythraceae alkaloids and homologs in croton oil- and carrageenan-induced inflammation

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PROTECTIVE EFFECTS OF CERTAIN LYTHRACEAE ALKALOIDS AND HOMOLOGS IN CROTON OIL- AND CARRAGEENAN-INDUCED INFLAMMATION

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INTRODUCTION

The Lythraceae plant family consists of 25 genera with approximately 580 species including Heimia salicifolia (H. B. K.) Link, Heimia myrtifolia, Cham. & Schl., and Decodon verticillatus, L. The first crystalline lythraceae alkaloids were described by Ferris (1), who isolated decaline, vertaline, decinine, decamine, decodine, verticillatine, and vertine from samples of Decodon verticillatus (Table I). Commonly called "water oleander" or "swamp loose-strife," this plant is found in moderate amounts along the Eastern seaboard of the United States and in swampy areas near Tallahassee, Florida. Blomster, et al. (2) reported the isolation of five alkaloids from Heimia salicifolia (lythrine, cryogenine, sinicuichine, heimine and sinine) and noted the presence of a phenolic group, two methoxy groups, a lactone function and aromatic unsaturation for each of these compounds (Table I). Douglas, et al. (3) compared the alkaloidal content of Heimia salicifolia and Heimia myrtifolia, showing that the two species have only

Table I. Alkaloids Isolated from Various Lythraceae Species

Alkaloid	Botanical Source	Chemical Formula	Melting Point	Reference
Lythrine	*Heimia salicifolia*	$C_{26}H_{29}NO_5$	241-243°C	2
Cryogenine	*Heimia salicifolia*	$C_{26}H_{29}NO_5$	253-255°C	2
Heimine	*Heimia salicifolia*	$C_{26}H_{29}NO_6$	247-249°C	2
Nesodine	*Heimia salicifolia*	$C_{26}H_{29}NO_5$	190°C	2,5
Lyfoline	*Heimia salicifolia*	$C_{26}H_{29}NO_5$	183°C	2,5
Sinicuichine	*Heimia salicifolia*	$C_{26}H_{29}NO_5$	187-188°C	2
Sinine (Lythridine)	*Heimia salicifolia*	$C_{26}H_{31}NO_6$	217-219°C	2,4
Decaline	*Decodon verticillatus*	$C_{26}H_{31}NO_5$	192-194°C	1
Vertaline	*Decodon verticillatus*	$C_{26}H_{31}NO_5$	194°C	1
Decinine	*Decodon verticillatus*	$C_{26}H_{31}NO_5$	222°C	1
Decamine	*Decodon verticillatus*	$C_{26}H_{31}NO_5$	222°C	1
Vertine (Cryogenine)	*Decodon verticillatus*	$C_{26}H_{29}NO_5$	245°C	1
Decodine	*Decodon verticillatus*	$C_{25}H_{19}NO_5$	193°C	1
Verticillatine	*Decodon verticillatus*	$C_{25}H_{27}NO_5$	312°C	1

slight variations in their alkaloidal composition. These authors further noted the exceedingly close botanical relationship between Heimia and Decodon; however, it was Blomster, et al. (2) who first published the empirical formula for cryogenine and noted its similarity to the Decodon alkaloid, vertine. Zacharias, et al. (4) determined cryogenine and vertine to be identical and established the structure of lythrine. The Heimia alkaloids, nesodine and lyfoline, have been fully characterized by Appel, et al. (5).

Heimia salicifolia (H.B.K.) Link is a small shrub which grows in the moist highlands of Mexico and northern South America and is used by the natives of these regions as a multipurpose medicinal and hallucinogen. Known as "herva de la vida" (herb of life), "abre-o-sol" (sun opener) and "sinicuiche" (twisted foot), the plant has a folklore reputation often bordering on the supernatural. The ingestion of a fermented decoction of the wilted leaves is reputed to induce a mild euphoria consisting of visual, auditory and perceptual distortions and a state of déjà vu whereby the partaker is able to remember with vivid clarity prenatal incidents (6). This fermented beverage is known in Mexico as "sinicuichi;" however, two other intoxicating plants, Rhynchosia praectoria, D.C. and Piscidia erythrina, L., also share this local nomenclature (7).

PHARMACOLOGICAL INVESTIGATIONS

The availability of pure alkaloids allowed Robichaud and Malone (8) to conduct the initial pharmacological investigations of Heimia salicifolia. These authors found that cryogenine appeared to account qualitatively and quantitatively for the action of whole-plant extracts and they concluded that cryogenine might possess "unique tranquilizing properties" since its action was devoid of the ataxic, blepharoptotic and autonomic side effects characteristically seen with reserpine and the phenothiazines. Additional work by these researchers (9) showed cryogenine to be active in reducing experimentally-induced anxiety in rats at doses which produce little or no side effects.

In 1966, Jiu (10) of G. D. Searle & Co. confirmed the central depressant activity of Heimia salicifolia extracts and found the plant to exhibit significant anti-inflammatory activity in two experimental models of inflammation. Jiu's observation, coupled with independent work at The University of Connecticut, led Kaplan, et al. (11) to conduct the first comprehensive anti-inflammatory evaluation of cryogenine. These experiments showed cryogenine to be almost as potent as phenylbutazone in preventing the development of artificially-induced inflammatory responses in rats using both acute (carrageenan pedal edema) and chronic (Mycobacterium butyricum adjuvant) models of inflammation. Cryogenine's

anti-inflammatory activity appeared unrelated to any toxic phenomena, and histopathological evaluations in rats after two weeks of daily oral dosing (100 mg/kg) revealed no lesions in any of the organs examined.

Cryogenine antagonizes several autacoids *in vitro*; however, this action generally appears nonspecific and can vary depending on the isolated tissue preparation utilized. Kinetic studies show cryogenine to be a non-competitive antagonist of acetylcholine (8, 9), and extremely high concentrations are required to block nervous transmission (9). While cryogenine is capable of reducing the pressor effects of exogenously-administered epinephrine in the anesthetized dog, its inability to antagonize epinephrine in the isolated rat *vas deferens* strongly supports the drug's lack of involvement with adrenergic receptors (12). Trottier and Malone (13) found cryogenine to competitively antagonize histamine in the isolated guinea pig uterus; however, this antagonism becomes non-competitive when the drug concentration is increased or when the isolated terminal ileum is used. A similar, non-competitive antagonism of serotonin was observed in the isolated rat uterus, and this finding correlates with cryogenine's ability to significantly reduce serotonin-induced pedal edema in the rat (11). Cryogenine also displays a mixed antagonism against synthetic bradykinnin (14). However, it is doubtful that its anti-inflammatory activity can be wholly attributed to its ability to non-specifically

antagonize autacoids at the local level and, based on its documented neuroleptic activity (8, 15), cryogenine's anti-inflammatory activity may have a centrally-mediated component.

The ability of certain centrally-acting drugs to possess anti-inflammatory activity is well established and related to their ability to (i) inhibit monoamine re-uptake and metabolism, (ii) alter peripheral hemodynamics, (iii) interfere with ganglionic transmission, or (iv) affect the pituitary-adrenal axis.

Structural and pharmacological similarities between cryogenine and tetrabenazine led Chang and Malone (16) to conclude that cryogenine does have a centrally-mediated anti-inflammatory component; neither of these two compounds affect liver glutathione stores nor alter peripheral catecholamine levels. While cryogenine does lower mean resting blood pressure and induce bradycardia in anesthetized dogs (12), these activities cannot explain its effect against chronic inflammation since it is well established that such hemodynamic alterations can play only a minimal role. Cryogenine neither facilitates nor inhibits ganglionic transmission (8, 17).

Cryogenine does affect the pituitary-adrenal axis but the nature of this involvement remains to be elucidated. The lack of thymic, adrenal, hepatic and hematic alterations in rats given oral doses (100 mg/kg/day) for 21 days first led

De Cato, et al. (18) to conclude that cryogenine's anti-inflammatory activity is probably not mediated by the endogenous release of corticosteroids; however, subsequent studies by these investigators (19) showed that crogenine's capacity to suppress both granuloma weight and carrageenan-induced pedal edema significantly diminished in bilaterally adrenalectomized rats. Therefore, cryogenine's anti-inflammatory activity may be due, in part, to the release of some adrenal principle. This hypothesis is supported by cryogenine's limited effectiveness in suppressing ultraviolet-induced erythema in guinea pigs (20), an experimental model of inflammation that is nonresponsive to corticosteroids and immunosuppressants.

Cryogenine's immunosuppressive activity was first evaluated by Kosersky, et al. (21) who, impressed by the drug's ability to inhibit both the irritant- and immune-mediated phases of adjuvant-induced polyarthritis (11), quantitatively confirmed this activity and clearly differentiated the action of cryogenine from that displayed by 6-mercaptopurine. A definitive study by Watson and Malone (22) confirmed cryogenine's lack of immunosuppressive capacity at effective anti-inflammatory dose levels.

The "water diuretic" property of certain lythraceae alkaloids was first reported by Weisbach (23) and further explored by Weibelhaus, et al. (24), who determined that lythrine and decinine, lythrine's dihydrocinnamic analog,

possessed significant activity. The failure of earlier investigators to document this capacity may be due to the relatively low content of lythrine found in Heimia salicifolia (25). Watson (26) found cryogenine to be devoid of any significant diuretic activity in the rat. Like prednisone, decinine has marked anti-inflammatory activity in several standard assays and restored diuresis in water-loaded, adrenalectomized rats (24). Unlike prednisone, decinine also blocks the anti-diuretic effects of vasopressin in both dogs and rats. Weibelhaus concluded:

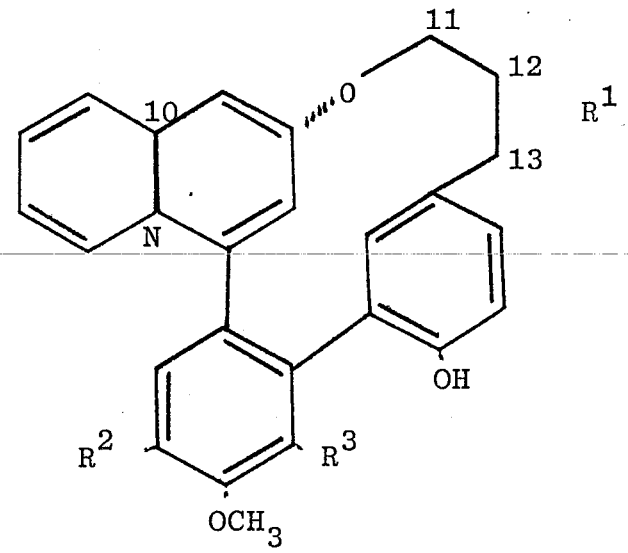
> "...while decinine can completely block the effects of ADH, it is probably not a specific antagonism and the compound may cause diuresis by acting at additional sites. Its glucocorticoid-like activity in adrenalectomized rats suggests an involvement with basement membrane permeability."

These data support glucocorticoid-like activity for certain of the lythraceae alkaloids and clearly demonstrate how relatively minor alterations in a complex molecular structure can greatly affect relative potencies and pharmacological activity.

CHEMICAL INVESTIGATIONS

There are four major groups of structurally-related lythraceae alkaloids (Table II) and, while all groups share the same basic skeleton, they differ in three specific regions: (i) the nature of the substituents and the pattern of substitution of the biphenyl ring system, (ii) the presence of a cinnamic or dihydrocinnamic lactone, and

Table II. Structurally-Related Lythraceae Alkaloids



Alkaloids	Quinolizidine Ring Fusion	R^1 12--13	R^2 C-4'	R^3 C-6'
Lythrine	trans	$-CH=CH-$	$-OCH_3$	H
Lyfoline	trans	$-CH=CH-$	-OH	H
Decinine	trans	$-CH_2-CH_2$	$-OCH_3$	H
Lythridine	trans	$-CHOH-CH_2-$	$-OCH_3$	H
Cryogenine	cis	$-CH=CH-$	$-OCH_3$	H
Decamine	cis	$-CH_2-CH_2-$	$-OCH_3$	H
Heimidine	cis	$-CHOH-CH_2-$	$-OCH_3$	H
Nesodine	trans	$-CH=CH-$	H	$-OCH_3$
Decodine	trans	$-CH_2-CH$ -	H	-OH
Dehydro-decodine	trans	$-CH=CH-$	H	-OH
Sinicuichine	cis	$-CH=CH-$	H	$-OCH_3$
Vertacillatine	cis	$-CH=CH-$	H	-OH
Dihydro-vertacillatine	cis	$-CH_2-CH_2-$	H	-OH

(iii) the stereo-chemistry at C-10 in the quinolizidine ring. The lythrine and cryogenine groups have oxygen substituents at the 4' and 5' positions on the biphenyl nucleus while the nesodine and verticillatine groups have oxygen substituents at the 5' and 6' positions. These groups can be divided further on the basis of their relative configurations at C-10 with a trans ring juncture for the nesodine and lythrine groups while the cryogenine and verticillatine groups have a cis ring juncture (27). Thus cryogenine and lythrine are configurational isomers (the same molecular formula, the same pattern of aromatic substitution, and the same configuration at the biphenyl linkage) and differ only in configuration within the quinolizidine ring system.

Ferris, et al. (28) first hypothesized that this characteristic quinolizidine ring system was derived by the conversion of lysine to delta'-piperidine with a subsequent transformation of the latter to isopelletierine, resulting in the formation of an asymetric center adjacent to the nitrogen atom. This pathway was firmly established by Spencer, et al. (29). Rother and Schwarting (30) found that both halves of the biphenyl ring system were derived from phenylalanine but the exact pathway by which phenylalanine enters the 4-phenylquinolizidine system still remains a mystery. Based on these reports, Rosazza, et al. (31) used a biogenetic approach in an attempt to synthesize dihydrofoline but were unsuccessful in the oxidative coupling of the two

phenyl rings. However, two independent groups (32, 33) were able to totally synthesize (±) decaline, a minor alkaloid of Decodon verticillatus, which differs from the previously mentioned alkaloids by having a biphenyl ether linkage (34). Other alkaloids in this group include methyldecaline, largerine, vertaline and demethylvertaline. To date, total syntheses of vertaline (35), methyldecinine (36) and methyldecamine (37), a biphenylquinolizidine, have been reported. Derivatives with a carbon skeleton of 4-phenylquinolizidine represent the fundamental structure of all lythraceae alkaloids (38) and are utilized as the key components in their syntheses. Two such phenylquinolizidines have been isolated from 5-10 day old seedlings of Heimia salicifolia but their absence from more mature plant samples suggests that they serve only as biosynthetic intermediates (39).

STATEMENT OF PROBLEM

The molecular complexity of the lythraceae alkaloids suggests that several active centers may account for their unique pharmacological profile. To assess these potentially active sites, two standard models of acute inflammation were selected for use in this present study -- the carrageenan-induced rat pedal edema assay and the croton oil-induced mouse ear edema assay.

The inflammatory response to an injection of carrageenan, a sulfated polysaccharide derived from Chondrus crispus

(Irish sea moss), was introduced by Winter, et al. (40) and developed by these workers into a laboratory model for use in the search for new anti-inflammatory agents (41, 42). This inflammogen gives more consistent results than other agents such as formalin, dextran and egg white (40) and potency results correlate very well with recommended clinical doses of many standard anti-inflammatory drugs (43).

The acute inflammatory reaction which results after the subplantar injection of carrageenan is characteristically biphasic. The first phase begins immediately upon injection, diminishes within one hour and accounts for 40 percent of the total edema. The second, more accentuated phase begins after one hour and persists through the third hour. Vinegar, et al. (44) determined that while aspirin will significantly inhibit both phases of edema formation, hydrocortisone is effective in suppressing only the second phase of swelling. Furthermore, the maximum suppression of edema formation by any one agent is only 70-75 percent. Van Arman concludes (45):

> "It is interesting that the upper limit of effectiveness of steroids and nonsteroids is approximately the same, but the mechanisms are different because one can achieve 100 percent inhibition by using, for example, indomethacin and dexamethasone together.

The second model utilized in this study is the croton oil-induced mouse ear edema assay, which was originally developed to determine the thymolytic and antiphlogistic

activity of topically-applied corticosteroids (46). However, Van Arman and co-workers (47, 48) have shown that this assay is also sensitive to several standard nonsteroidal anti-inflammatory agents. Furthermore, the relative potencies determined by this assay correlate well with established clinical potencies. Since the assay is rapid, reliable, and requires only modest amounts of test compounds, it is especially well suited for natural products research, where it is often difficult to obtain large amounts of pure principles for pharmacological testing.

While the oral anti-inflammatory capacity of several of the lythraceae alkaloids has been well documented, their topical antiphlogistic capacity has not yet been evaluated. Moreover, the specific function or functions of the molecule which account for this anti-inflammatory capacity remain a mystery. The present study was undertaken: (i) to assess the topical anti-inflammatory potential of cryogenine, lythrine and two selected lythraceae intermediates and (ii) to investigate the possible molecular components which produce this established, yet enigmatic anti-inflammatory effect.

MATERIALS AND METHODS

Isolation of the Alkaloids. -- Four kilograms of a methanol extract of dried and defatted Heimia salicifolia (prepared by W. C. Watson) were dissolved in 10 liters of 2 N HCl and centrifugally filtered through three layers of filter paper (Filtrax, coarse grade). The solid residue was redissolved in another 10 liters of 2 N HCl and continually stirred for seven days prior to filtration in the manner described above. The non-alkaloidal, nonpolar principles were then removed by extracting aliquots of the combined acidified filtrates with equal volumes of diethyl ether in a glass separatory funnel until the ether phase was clear and tested negative for alkaloids. The aqueous phase was titrated to a pH of 9.0 by 58 percent ammonium hydroxide, yielding a rust-colored suspension. Aliquots of this basified, aqueous fraction were combined with equal volumes of chloroform in a glass separatory funnel and extracted until exhausted of all alkaloids. The resulting 60 liters of chloroform extract were filtered by gravity through sodium sulfate to remove

residual water, then dried *in vacuo* at 40°C. The resulting dried chloroform extract (150 g) represented an approximate 4 percent yield from the methanol extract.

The dried chloroform extract was reduced to a fine powder in a glass mortar, dissolved in a minimum amount of absolute methanol and filtered through Whatman #1 filter paper in a Buchner funnel under suction. The filtrate was added in increments to a warm evaporating dish containing 100 g of aluminum oxide (active neutral, activity I). Residual methanol was evaporated at 40°C *in vacuo*, leaving an admixture of chloroform extract and aluminum oxide at a ratio of 3:2. The admixture was sprinkled onto a Bio-Rad glass column (4.5 cm x 65 cm) containing 650 g of aluminum oxide dry packed with benzene. The chloroform extract to aluminum oxide column packing represented a ratio of 4:1. Elution was accomplished using the following sequence of solvents: (*i*) benzene, 100 ml; (*ii*) benzene:chloroform 95:5, 10 liters; (*iii*) benzene:chloroform 50:50, 20 liters; and (*iv*) chloroform:methanol 50:50, 3 liters.

The first three fractions were combined and reduced by flash evaporation at 40°C *in vacuo*. The resultant green-yellow precipitate was dissolved in a minimum amount of boiling ethyl acetate and filtered through Whatman #1 filter paper in a Buchner funnel under suction. The filtrate was then heated to clarity and petroleum ether was added dropwise until the mixture turned slightly opaque. The mixture

was again heated to clarity, covered and placed in a dark desiccator at room temperature for 24 hours. The resulting white crystals were collected on Whatman #1 filter paper in a Buchner funnel under suction. The mother liquor was then reduced to half volume with a current of air and crystallization allowed to occur as described above. This procedure was repeated until no further crystals formed. Thin-layer chromatographic (TLC) analysis showed these crystals to contain a mixture of lythrine, cryogenine and an unidentified yellow pigment.

A separation procedure differing from the original method of Blomster, et al. (2) was attempted based on preliminary analytical-scale experiments which gave selective elution of lythrine and cryogenine by decreasing the benzene to chloroform ratio in five percent increments. At a 70:30 ratio of benzene to chloroform, a pure lythrine fraction was eluted to exhaustion. A further decrease in the benzene to chloroform ratio (60:40) yielded a pure cryogenine fraction. Both lythrine and cryogenine fractions were monitored by TLC. However, these results could not be duplicated when attempted on a preparative scale, so the following high pressure liquid chromatographic separation was developed.

Analytical samples of six Heimia alkaloids were dissolved (1.0 mg/ml) in methanol:methylene chloride (70:30). Individual retention times were determined by injecting 10 microliters of the alkaloidal solutions onto a column using

a Rheodyne syringe-loading sample injector fitted with a 175-microliter sample loop. The mobile phase (methanol: methylene chloride 70:30) was filtered through a 0.22 micron Millipore filter under suction and then pumped at a rate of 2.0 ml/minute by a Perkin Elmer Series 2/1 HPLC. Separation was accomplished using a Partisil SAX 10 analytical anion-exchange column. *Heimia* alkaloids were detected with a Perkin Elmer LC-55 spectrophotometer set at 285 nanometers and monitored with an Omni-Scribe chart recorder. The column was allowed to equilibrate for two hours prior to use and all separations were performed at room temperature (72-75^{o}F). Retention times for the six *Heimia* alkaloids are listed in Table III.

High pressure liquid chromatographic (HPLC) separation of cryogenine and lythrine on a preparative scale was then carried out as follows. The mixed alkaloidal fraction obtained by column chromatography and corresponding to "Fraction I" of Blomster, *et al*. (2) was dissolved in 1.0 ml of methanol:methylene chloride (70:30) and passed through a Sep-Pak cartridge silica filter to remove any highly polar and particulate principles. The cartridge was flushed with 4.0 ml of solvent to insure complete elution of the alkaloids. The filtrate was reduced to dryness by flash evaporation at 40^{o}C *in vacuo* then re-dissolved in 2.0 ml of methanol: methylene chloride (70:30). A 1.0 ml aliquot was injected utilizing a Rheodyne sample injector fitted with a 1.1-ml sample loop.

Table III. Retention Times for Six Lythraceae Alkaloids Determined by HPLC

Alkaloid[a]	Quantity, mcg	Retention Time, sec.	Minor Peaks, sec.
Lythrine	10	100	40, 50
Lythridine	10	105	40, 600
Nesodine	10	115	40, 50
Lyfoline	10	170	40, 115, 600
Sinicuichine	50	450	40, 50, 95, 125
Cryogenine	10	605	40, 140, 220, 415

[a]All alkaloids used for analytical HPLC determinations were extracted from Heimia salicifolia by A. E. Schwarting and A. Rother at The University of Connecticut, Storrs CT.

The mobile phase (methanol:methylene chloride 70:30) was pumped at a rate of 3.0 ml/minute by a Perkin Elmer Series 2/1 HPLC through a Whatman Partisil 10 SAX M-9 preparative column (9.4 mm x 500 mm) and passed through a Perkin Elmer LC-55 spectrophotometer set at 250 nanometers. The fractions were monitored by a Houston Instrument Omni-Scribe chart recorder set at 0.1 volts with a chart speed of 0.25 cm/minute and collected in 4.0-ml fractions. At a sample concentration of 50 mg/ml, the resolution of the two alkaloidal peaks was quite distinctive. Identification of the peaks was determined by TLC. Lythrine eluted well in advance of cryogenine.

The cryogenine fractions collected from the apex of its elution peak were combined and flash evaporated at 40°C *in vacuo*, leaving a white precipitate. To insure that the cryogenine was in the free-base form, this precipitate was dissolved in 25 ml of 10 percent acetic acid and brought to a pH of 9.0 by the drop-wise addition of 58 percent ammonium hydroxide. The basic aqueous solution was placed in a glass separatory funnel and extracted with chloroform until further additions of chloroform tested negative for cryogenine by TLC. The chloroform fraction was then flash evaporated at 40°C *in vacuo* leaving a white precipitate which was dissolved in a minimum amount of boiling absolute methanol, covered and placed overnight in a dark desiccator at room temperature. The resulting crystals were collected

on Whatman #1 filter paper using a Buchner funnel under suction and washed three times with cold, absolute methanol. The crystals were dried at room temperature *in vacuo* over phosphorous pentoxide for 24 hours. This method of isolation yielded cryogenine base of high purity as determined by melting point, two-dimensional TLC and infra-red spectrophotometry. The lythrine fractions were treated in the same manner with similar results.

Carrageenan Pedal Edema in the Rat. -- Four synthetic phenylquinolizidine compounds prepared by Dr. James Quick of Northeastern University (Figure 1) were screened in rats for potential anti-inflammatory activity using the carrageenan pedal edema assay proposed by Winter, *et al*. (40) and modified by Van Arman, *et al*. (49). Adult, healthy, Sprague-Dawley derived male rats (150-170 g; Simonsen Laboratories, Gilroy CA) were maintained in our laboratory for one week after receipt with free access to Purina Laboratory Chow and tap water. Twenty-four hours prior to experimentation, the animals were placed on fast (tap water *ad libitum*) and housed in individual cages with wide-mesh screen floors to prevent coprophagy. Thirty minutes prior to the carrageenan injection, the animals were dosed in a random pattern with either a test drug finely suspended in 0.25 percent agar or the 0.25 percent agar dosing vehicle alone. Oral administration was selected to avoid any parenteral "counterirritant"

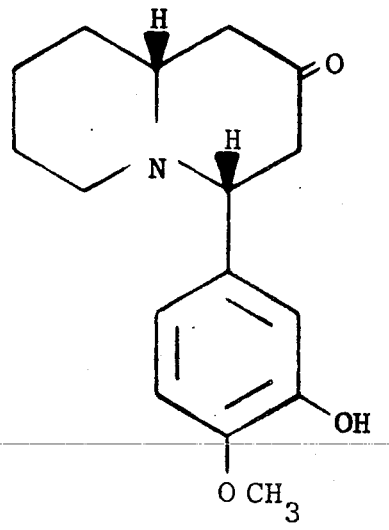
Figure 1. Synthetic Intermediates Prepared by Dr. James B. Quick.

RR-5-2.....Octahydro-4-(3-hydroxy-4-methoxyphenyl) -2H-quinolizin-2-one; m.p. = 138-140° [Hexane-Benzene]; light tan-white; 1 g.

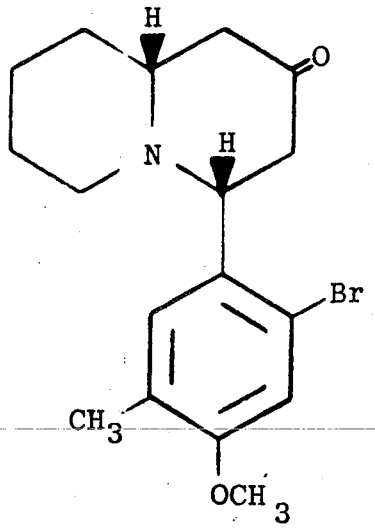
JB-1-0.....4-(2-bromo-4,5-dimethoxyphenyl) -octahydro-2H-quinolizin-2-one; m.p. = 136-138° [Hexane]; light yellow-tan; 700 mg.

MM-1-0.....Octahydro-4-(3,4-dimethoxyphenyl)-2H-quinolizin-2-one; viscous liquid; golden brown; 500 mg.

HR-1-0.....3-(4-hydroxyphenyl)-propenoic acid octahydro-4-(3-hydroxy-4-methoxyphenyl) -2H-quinolizin-2-yl ester; viscous liquid; light golden brown; 500 mg.



RR-5-2



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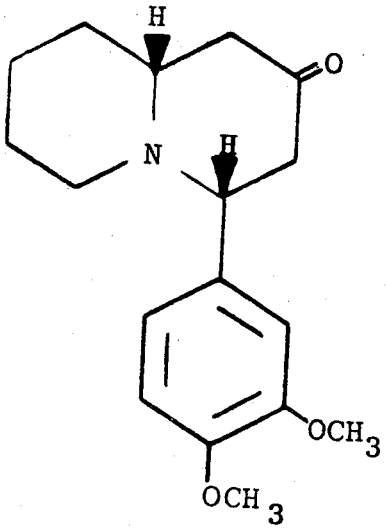

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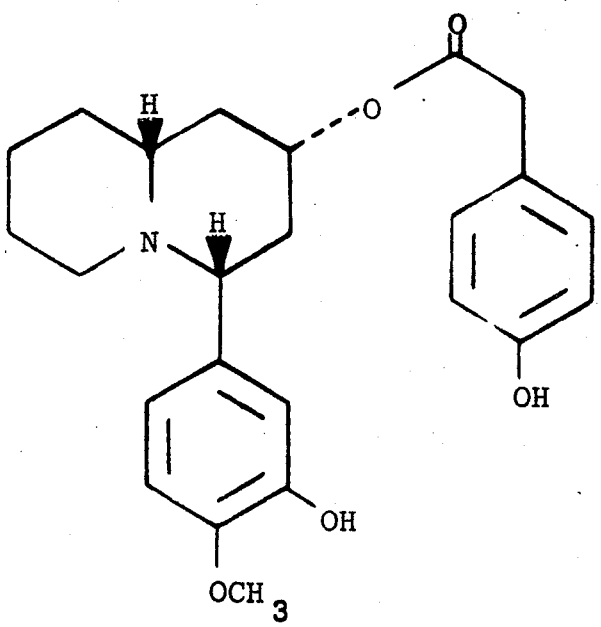


HR-1-0

effect which might modify the development of the pedal edema. Five minutes before carrageenan injection, the volume of the right hind paw was measured plethysmographically to a line circumscribed just above the animals's tibio-carpal joint. At time zero, a subplantar injection of 0.1 ml of 1.0 percent carrageenan in sterile 0.9 percent saline was made in the right hind paw utilizing a micrometer syringe with a 27-gauge 3/4-inch needle. The control group received an equivalent amount of sterile 0.9 percent saline. Paw volumes were determined as previously described at +5 and +30 minutes and at half-hour intervals thereafter. Tests for significant inhibition of carrageenan-induced pedal edema by the test compounds were made utilizing analysis of variance statistical techniques.

Croton Oil Mouse Ear Edema Assay. -- The topical anti-inflammatory activities of selected natural and synthetic compounds were determined using the croton oil mouse ear edema assay of Tonelli, et al. (46) as modified by Van Arman (47). Adult, healthy Swiss-Webster derived female mice (17-20 g; Simonsen Laboratories, Gilroy CA) were housed in our facilities for at least one week prior to use and given Purina Laboratory Chow and tap water ad libitum. One hour prior to experimentation, the mice were randomly placed in individual cages with wide-mesh screen floors to prevent coprophagy and provided free access to food and tap water

for the remainder of the experiment. Each mouse was anesthetized with ether and, upon loss of righting reflex, 0.1 ml of a solution containing 2 percent croton oil, 73 percent ether, 20 percent pyridine and 5 percent double-distilled water was applied in four aliquots using a 25-microliter Eppendorf pipette (two applications to the anterior and two to the posterior surfaces of each animal's right ear).

One hour after the application of the croton oil solution, the individual test compounds were dissolved or suspended at concentrations containing the specified drug dose in 0.1 ml of a vehicle consisting of 75 percent ether, 20 percent pyridine and 5 percent double-distilled water. The mice were again anesthetized with ether and 0.1 ml of drug solution/suspension applied to the right ear in the manner previously described. Vehicle alone was applied to the ears of the control animals.

Three hours after the drug applications, the mice were sacrificed with ether and both the right and left ears excised at the base. A 8.0-mm circle of ear tissue was removed with a size #4 cork borer, using the outer margin of the ear as a landmark in order to avoid the extra hair and tissue found at the base of the ear. The sections were placed in marked spot-test plates to insure proper identification and then individually weighed to the nearest tenth of a milligram on a Sartorius laboratory balance.

The increase in weight induced by croton oil was found

by subtracting the weight of the untreated left ear section from that of the right ear section. Drug effects (expressed as percent inhibition) were determined by subtracting the weight increase of treated individual ears from the mean weight increase of the control group and the difference times 100 divided by the mean weight increase of the controls. Analysis of variance statistical techniques were used to determine the significance of any inhibition of the croton oil-induced ear edema.

RESULTS

Carrageenan-Induced Rat Pedal Edema Assay. -- The subplantar injection of carrageenan resulted in the characteristic biphasic acute inflammatory response (Table IV, Figure 2). Orally administered aspirin (400 mg/kg) significantly ($P < 0.05$) inhibited the acute inflammatory reaction at all time intervals evaluated (Table IV). Indomethacin given orally (12.5 mg/kg) displayed noticeable anti-inflammatory activity; however, this activity became significant ($P < 0.05$) only during the final half hour of the five-hour evaluation (Table IV, Figure 3). Indomethacin's lack of activity in suppressing the first phase of carrageenan-induced pedal edema is well established (44); however, its delayed onset during the present study may be due, in part, to the interval between drug dosing and carrageenan injection which was shortened by one-half hour to facilitate the number of compounds evaluated.

Table IV. Effects of Seven Orally Administered Selected Drugs on Carrageenin-induced Rat Pedal Edema

Parameter	Mean ± 1 SEM (Significance Level Injection Control (N = 7)	Carrageenin Control (N = 10)	Aspirin, 400 mg/kg (N = 10)
Whole Body Weight, g	155.6±9.4 (> 0.50)	162.3±5.9	159.0±5.0 (> 0.60)
Paw Volume, ml, -5 min.	0.92±0.05 (> 0.60)	0.89±0.02	0.88±0.03 (> 0.70)
Paw Volume, ml +5 min.	1.08±0.05 (> 0.90)	1.08±0.04	1.06±0.04 (> 0.70)
Volume Change From +5 min			
+30 min, ml	-0.01±0.01 (< 0.001)*	+0.18±0.04	+0.05±0.3 (< 0.02)*
+60 min, ml	-0.05±0.02 (< 0.01)*	+0.32±0.08	+0.01±0.04 (< 0.05)*
+90 min, ml	-0.05±0.03 (< 0.01)*	+0.33±0.08	+0.11±0.04 (< 0.02)*
+120 min, ml.	-0.06±0.02 (< 0.001)*	+0.31±0.07	+0.09±0.04 (< 0.02)*
+150 min, ml	-0.07±0.03 (< 0.001)*	+0.37±0.08	+0.12±0.04 (< 0.01)*
+180 min, ml	-0.05±0.02 (< 0.001)*	+0.43±0.08	+0.14±0.04 (< 0.01)*
+210 min, ml	-0.06+0.03 (< 0.001)*	+0.47+0.09	+0.17+0.05 (< 0.01)*

vs. Carrageenin Control, P)[a]

Indomethacin, 12.5 mg/kg (N = 10)	RR-5-2, 100 mg/kg (N = 10)	JB-1-0, 100 mg/kg (N = 10)	MM-1-0 100 mg/kg (N = 10)	HR-1-0, 100 mg/kg (N = 10)
164.0±5.3 (> 0.80)	164.5±5.0 (> 0.70)	160.4±4.9 (> 0.80)	164.0±5.5 (> 0.80)	160.2±6.4 (> 0.80)
0.94±0.03 (> 0.20)	0.97±0.05 (> 0.10)	0.86±0.03 (> 0.40)	0.92±0.03 (> 0.40)	0.88±0.04 (> 0.80)
1.10±0.03 (> 0.60)	1.14±0.05 (> 0.20)	1.01±0.03 (> 0.10)	1.10±0.03 (> 0.60)	1.05±0.04 (> 0.50)
+0.14±0.03 (> 0.30)	+0.19±0.05 (> 0.80)	+0.10±0.02 (> 0.05)	+0.18±0.02 (> 0.90)	+0.17±0.04 (> 0.80)
+0.25±0.07 (> 0.40)	+0.32±0.09 (> 0.90)	+0.17±0.03 (> 0.05)	+0.33±0.04 (> 0.90)	+0.31±0.06 (> 0.90)
+0.27±0.07 (> 0.50)	+0.30±0.08 (> 0.70)	+0.19±0.03 (> 0.10)	+0.40±0.05 (> 0.40)	+0.34±0.07 (> 0.90)
+0.29±0.07 (> 0.80)	+0.30±0.07 (> 0.80)	+0.20±0.03 (> 0.10)	+0.40±0.05 (> 0.30)	+0.36±0.06 (> 0.60)
+0.30±0.07 (> 0.50)	+0.32±0.06 (> 0.60)	+0.21±0.03 (> 0.05)	+0.40±0.05 (> 0.70)	+0.38±0.06 (> 0.80)
+0.32±0.07 (> 0.30)	+0.38±0.07 (> 0.60)	+0.23±0.03 (< 0.05)*	+0.47±0.07 (> 0.70)	+0.42±0.06 (> 0.90)
+0.29±0.05 (> 0.10)	+0.42±0.09 (> 0.70)	+0.27±0.04 (< 0.05)*	+0.48±0.06 (> 0.80)	+0.47±0.07 (> 0.90)

Table IV (continued)

Parameter	Mean ± 1 SEM (Significance Level)		
	Injection Control (N = 7)	Carrageenin Control (N = 10)	Aspirin, 400 mg/kg (N = 10)
Volume Change From +5 min			
+240 min, ml	-0.06±0.03 (< 0.001)*	+0.48±0.09	+0.13±0.05 (< 0.01)*
+270 min, ml	-0.05±0.03 (< 0.001)*	+0.50±0.08	+0.12±0.05 (< 0.01)*
+300 min, ml	-0.05±0.03 (< 0.001)*	+0.51±0.08	+0.10±0.04 (< 0.001)*

[a]Asterisks have been added to emphasize statistical significance.

vs. Carrageenin Control, P)[a]

Indomethacin, 12.5 mg/kg (N = 10)	RR-5-2, 100 mg/kg (N = 10)	JB-1-0, 100 mg/kg (N = 10)	MM-1-0 100 mg/kg (N = 10)	HR-1-0, 100 mg/kg (N = 10)
+0.29±0.05 (> 0.05)	+0.45±0.10 (> 0.80)	+0.29±0.04 (> 0.05)	+0.55±0.06 (> 0.50)	+0.50±0.07 (> 0.80)
+0.27±0.05 (< 0.05)*	+0.46±0.10 (> 0.70)	+0.30±0.05 (> 0.05)	+0.54±0.06 (> 0.60)	+0.51±0.08 (> 0.90)
+0.25±0.06 (< 0.02*	+0.50±0.11 (> 0.90)	+0.33±0.05 (> 0.05)	+0.58±0.06 (> 0.40)	+0.52±0.07 (> 0.90)

Figure 2. Time Course of Carrageenan-Induced Pedal Edema in the Rat.

x = Carrageenan Control

o = Aspirin (400 mg/kg)

Δ = JB-1-0 (100 mg/kg)

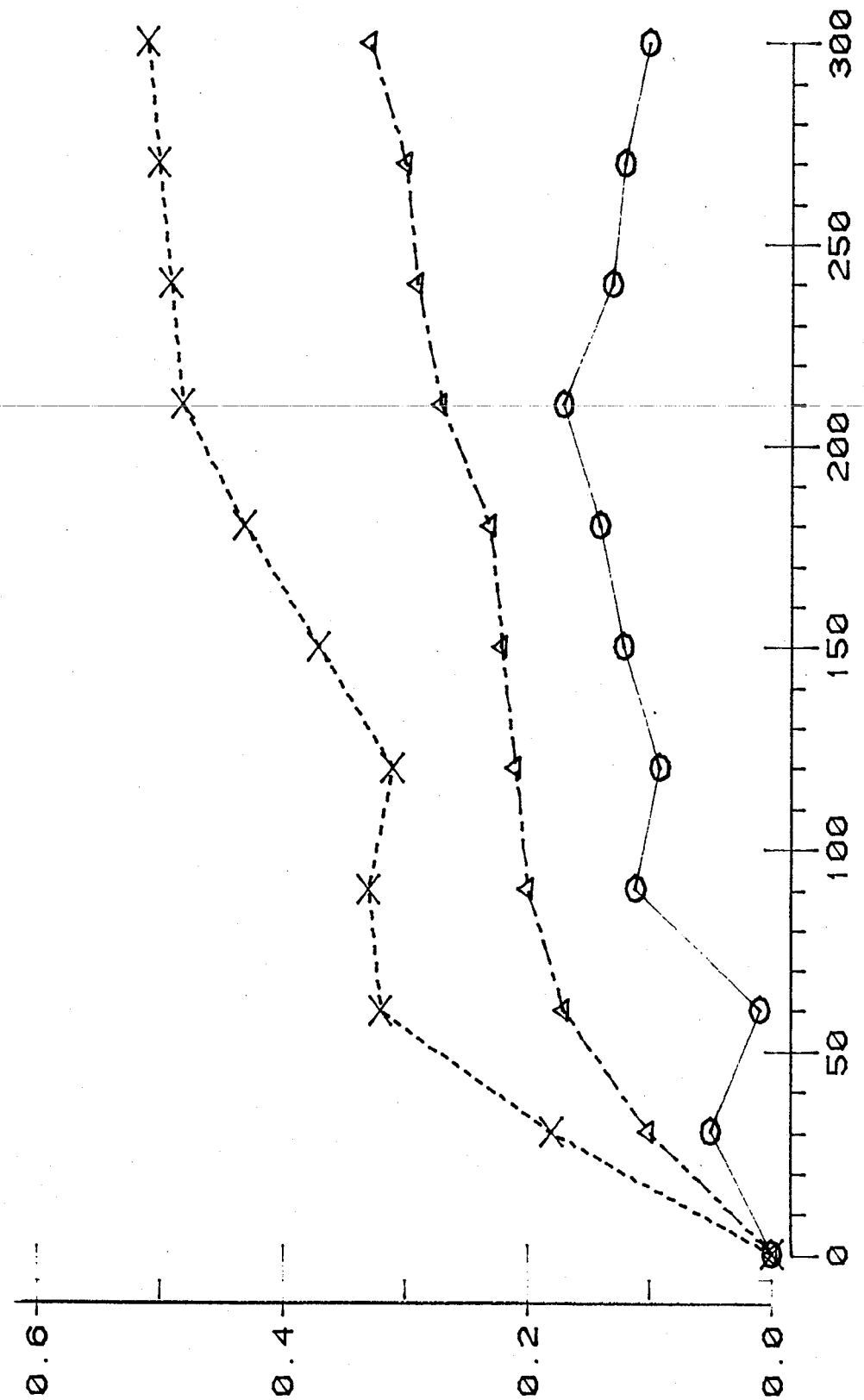
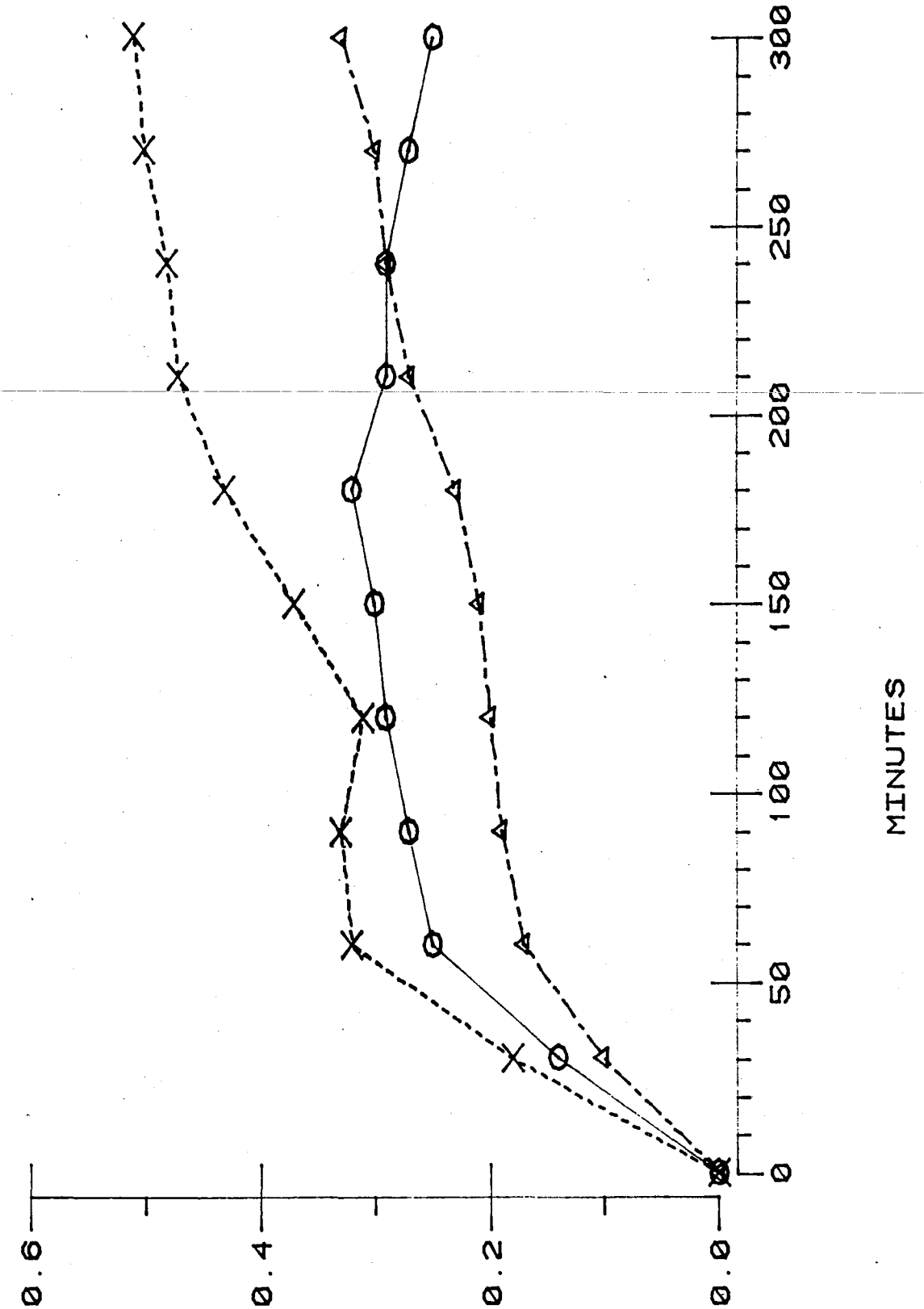

0.0
0.2
0.4
0.6
0
50
100
150
200
250
300
MINUTES
ΔOD AT 340 NM

Figure 3. Time Course of Carrageenan-Induced Pedal Edema in the Rat.

x = Carrageenan Control

o = Indomethacin (12.5 mg/kg)

Δ = JB-1-0 (100 mg/kg)


0.0
0.2
0.4
0.6
0
50
100
150
200
250
300
MINUTES

JB-1-0 showed significant ($P < 0.05$) anti-inflammatory activity at times +180 through +210 minutes (Table IV, Figure 2) when given orally at a dose of 100 mg/kg. At this dose level, a marked sedation was noted in all animals screened. This peculiar sedation, which began two hours after the compound was administered and persisted throughout the course of the experiment, is noteworthy since all prior reports of the depressant and ataractic properties of lythraceae alkaloids were achieved only when the compounds were administered by intraperitoneal injection. Three additional synthetic phenylquinolizidines, RR-5-2 (Figure 4), MM-1-0 (Figure 5) and HR-1-0 (Figure 6) were essentially devoid of any real anti-inflammatory activity when given orally at a dose of 100 mg/kg. --this dosage of cryogenine will consistently suppress the second stage inflammatory response of carrageenan pedal edema (11, 19, 21).

Croton Oil-Induced Mouse Ear Edema Assay.--The topical application of croton oil produced a significant increase in ear weight characterized by edema, hyperemia and an overall increase in ear size. Table V and Figures 7 - 9 depict the dose-response data (8 mice per dose) used to calculate the respective potencies and ID_{50} (mg/ear to produce the 50 percent suppression of edema) values that are summarized in Table VI. An assay lambda (s/b) could not be calculated for the assay of dexamethasone vs indomethacin since the

Figure 4. Time Course of Carrageenan-Induced Pedal Edema in the Rat.

x = Carrageenan Control

o = RR-5-2 (100 mg/kg)

Δ = JB-1-0 (100 mg/kg)

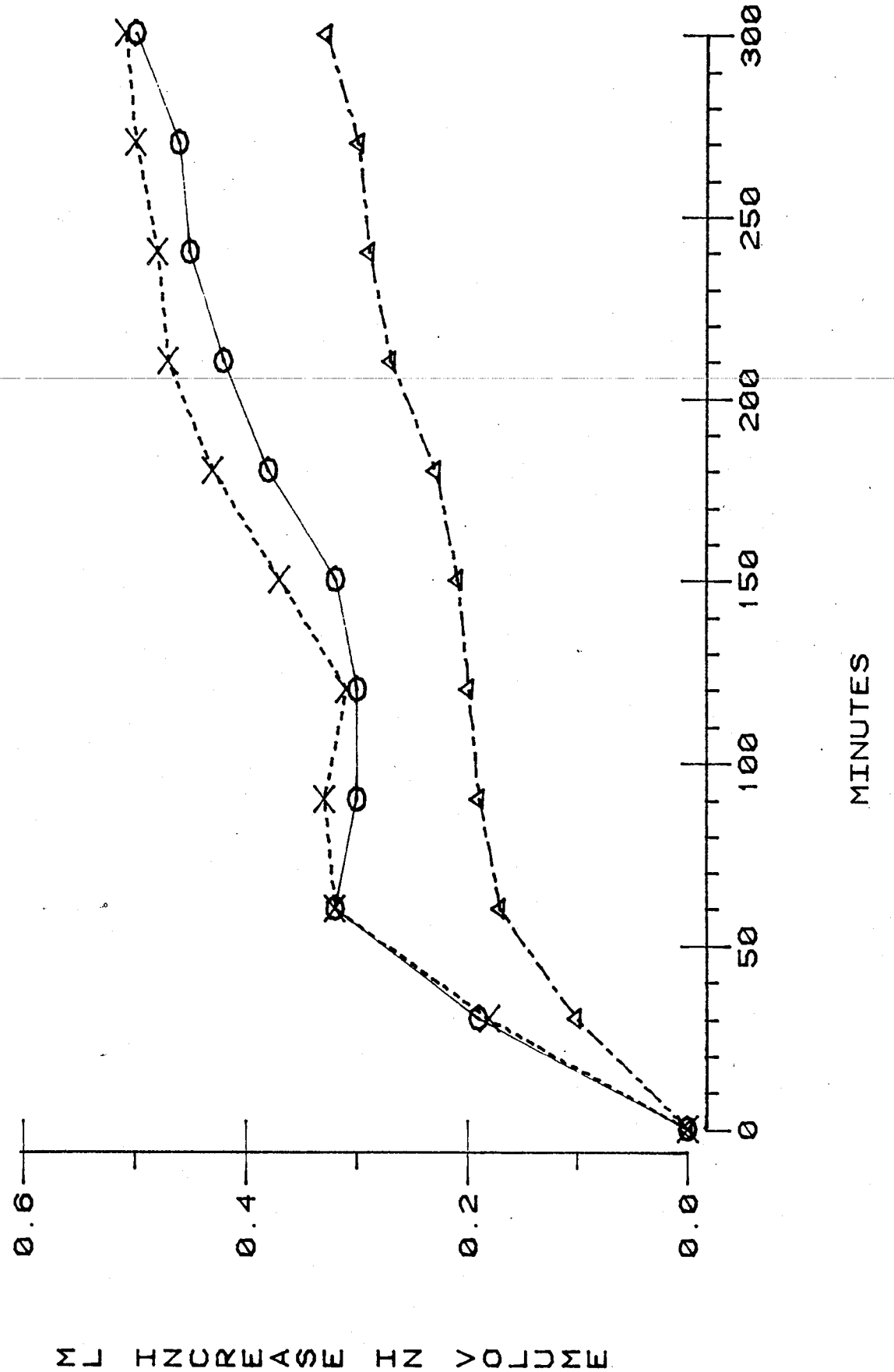

0.0
0.2
0.4
0.6
0
50
100
150
200
250
300
MINUTES

Figure 5. Time Course of Carrageenan-Induced Pedal Edema in the Rat.

x = Carrageenan Control

Ω = MM-1-0 (100 mg/kg)

Δ = JB-1-0 (100 mg/kg)

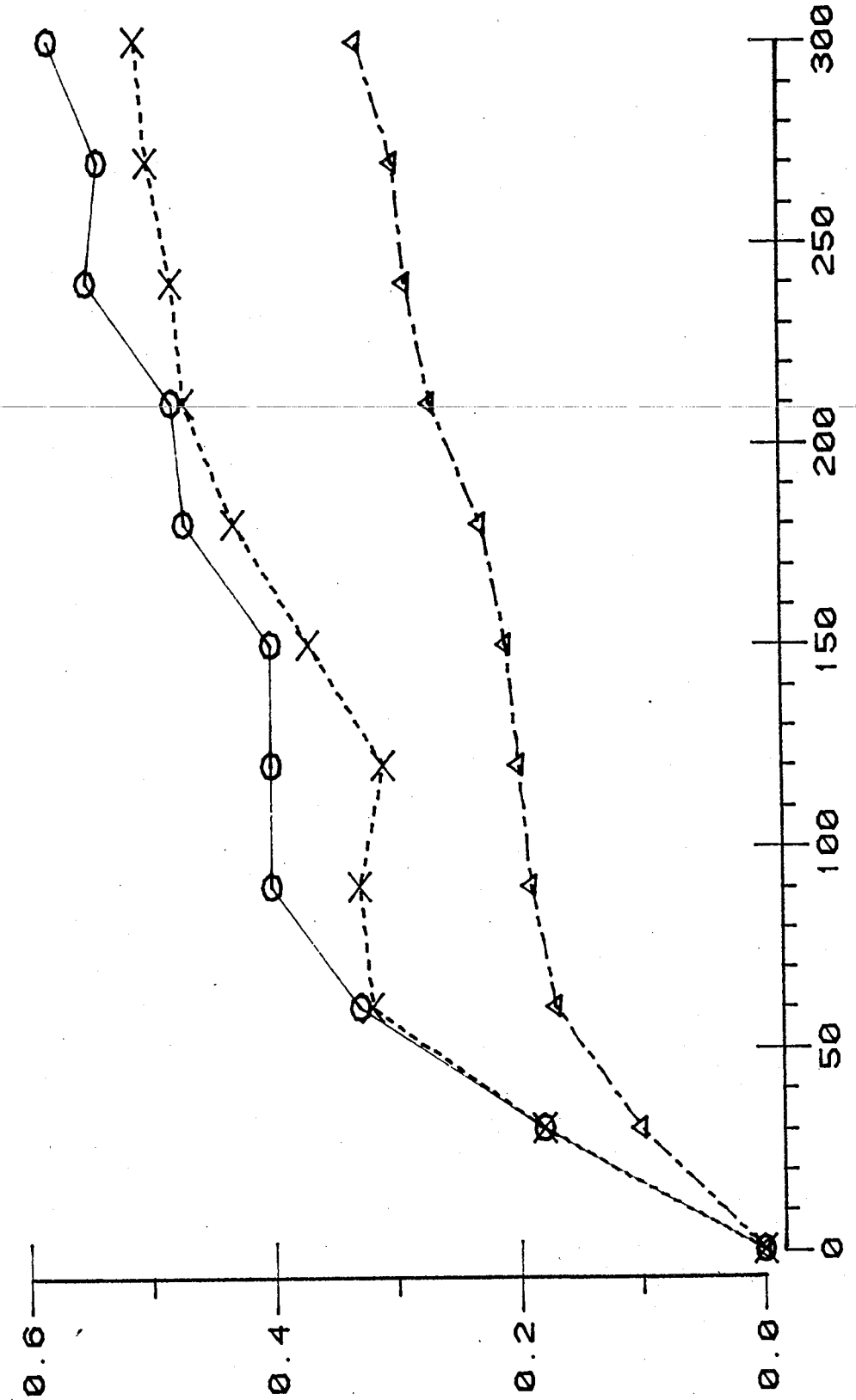

0.0
0.2
0.4
0.6
0
50
100
150
200
250
300
MINUTES
ΔCETOX ZH ASYRAZH FZ

Figure 6. Time Course of Carrageenan-Induced Pedal Edema in the Rat.

x = Carrageenan Control

α = HR-1-0 (100 mg/kg)

Δ = JB-1-0 (100 mg/kg)

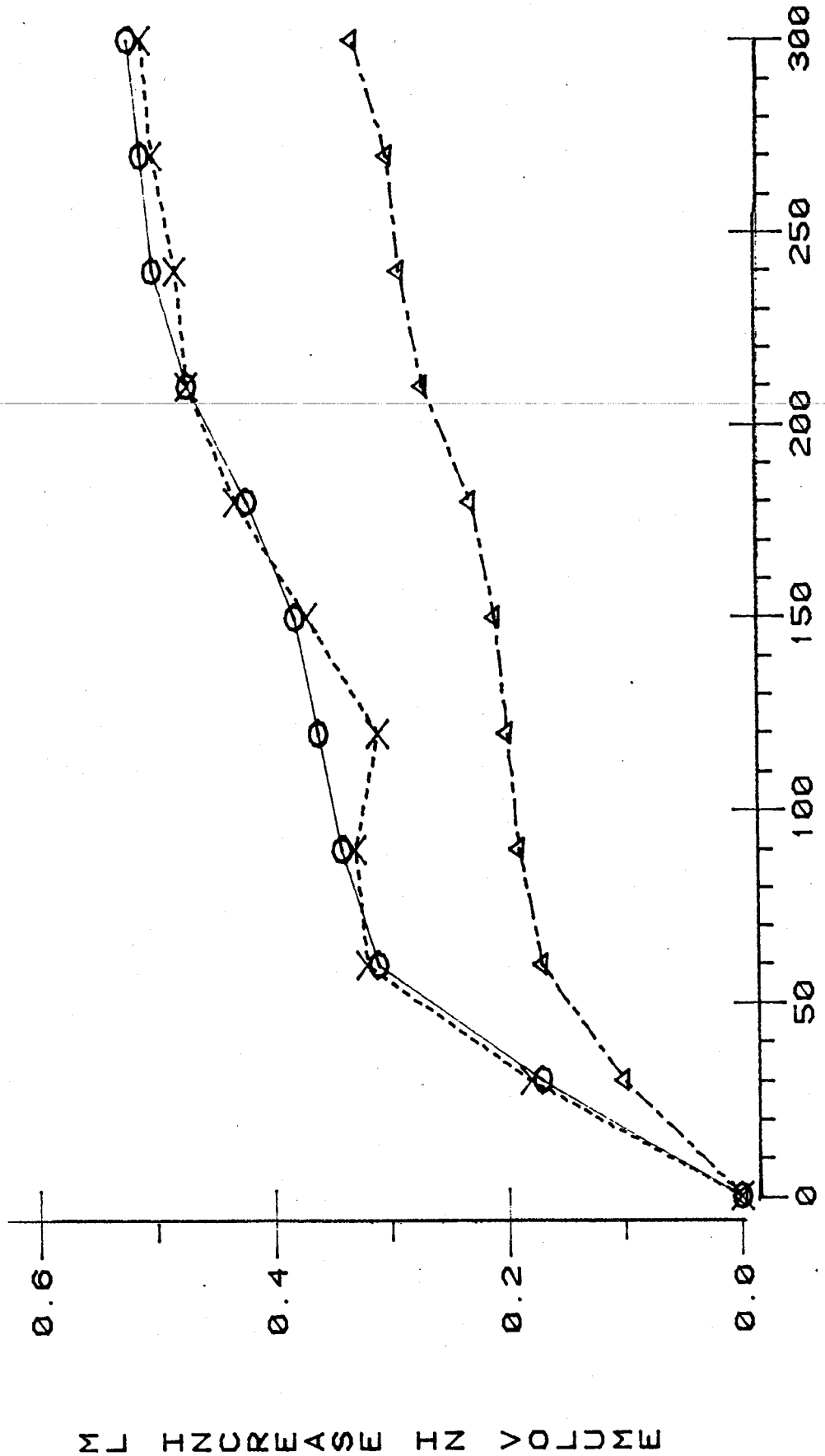

0.0
0.2
0.4
0.6
0
50
100
150
200
250
300
MINUTES

Table V. Drug Effects on Croton Oil-Induced Mouse Ear Edema

Treatment	Drug (mg/ear)	N	Mean Values (SEM) $W_t - W_c$	Percent Inflammation	Percent Inhibition
Control	0	11	18.74 (0.84)	130.27 (5.27)	0
Indomethacin	0.5	8	12.14 (1.56)	79.50 (10.36)	36.75 (7.13)
	1.0	8	9.13 (1.11)	60.00 (8.58)	51.13 (5.98)
	2.0	8	5.08 (0.26)	35.25 (1.85)	73.00 (1.38)
Phenylbutazone	2.0	8	11.81 (0.70)	79.13 (5.73)	37.13 (3.71)
	4.0	8	7.75 (1.24)	52.75 (8.36)	58.63 (6.58)
	8.0	8	6.05 (0.84)	41.38 (5.94)	67.63 (4.43)
Aspirin	1.0	8	10.45 (1.06)	77.00 (8.49)	44.25 (5.65)
	2.0	8	6.19 (0.81)	48.50 (6.83)	67.00 (4.35)
	4.0	8	3.08 (0.39)	22.88 (3.13)	83.63 (2.14)
Dexamethasone	0.0001	8	10.50 (0.98)	79.13 (8.51)	44.13 (5.29)
	0.001	8	8.04 (0.98)	64.75 (9.21)	57.38 (5.20)
	0.01	8	4.68 (0.99)	38.00 (5.84)	73.13 (4.33)

Table V. Continued

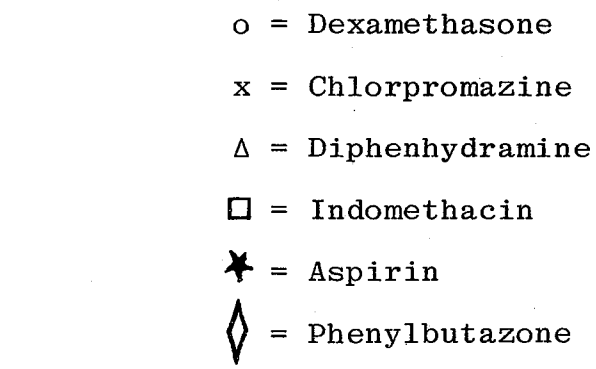
Treatment	Drug (mg/ear)	N	Mean Values (SEM) W_t-W_c	Percent Inflammation	Percent Inhibition
Cryogenine	0.25	8	13.80 (2.07)	95.50 (14.43)	31.00 (8.50)
	0.50	8	9.70 (1.23)	67.75 (8.57)	48.25 (6.56)
	1.0	8	6.68 (1.61)	44.88 (9.77)	64.50 (8.61)
Lythrine	0.25	8	11.69 (1.38)	97.25 (11.60)	37.75 (7.34)
	0.50	8	8.46 (0.84)	69.75 (7.21)	54.75 (4.51)
	1.0	8	5.61 (0.57)	49.13 (4.93)	70.00 (3.04)
JB-1-0	0.25	8	10.83 (1.20)	78.63 (8.82)	42.25 (6.39)
	0.50	8	6.33 (1.12)	45.13 (7.96)	66.38 (6.00)
	1.00	8	3.81 (0.55)	25.75 (3.97)	79.63 (2.93)
p-Hydroxy-cinnamic acid	1.0	8	10.73 (1.24)	93.88 (8.95)	42.75 (6.58)
	2.0	8	7.90 (1.58)	79.63 (16.2)	57.75 (8.46)
	4.0	8	4.50 (0.87)	46.50 (9.63)	76.00 (4.64)

Table V. Continued

Treatment	Drug (mg/ear)	N	Mean Values (SEM)		
			$W_t - W_c$	Percent Inflammation	Percent Inhibition
Chlorpromazine	0.015	8	10.83 (1.20)	75.75 (9.79)	43.50 (6.73)
	0.031	8	8.38 (1.46)	59.00 (10.34)	55.25 (7.81)
	0.062	8	6.69 (0.88)	46.38 (5.62)	64.25 (4.69)
Diphenhydramine	0.038	8	12.05 (1.22)	91.25 (9.29)	35.75 (6.45)
	0.075	8	7.59 (1.08)	55.88 (7.73)	63.25 (6.07)
	0.15	8	4.25 (0.89)	42.00 (8.92)	70.75 (5.93)
Cinnamic Acid	1.0	8	18.84 (2.26)[a]	123.25 (13.47)	13.75 (6.34)
	2.0	8	13.75 (1.85)	89.50 (11.81)	29.88 (8.21)
	4.0	8	12.15 (1.64)	85.88 (11.10)	36.50 (7.84)

[a]Value does not differ from Control ($P < 0.05$ by Student's t test)

Figure 7. Log Dose-Response Curve for Six Reference Compounds in Regard to the Suppression of Croton Oil-Induced Edema in Mice.



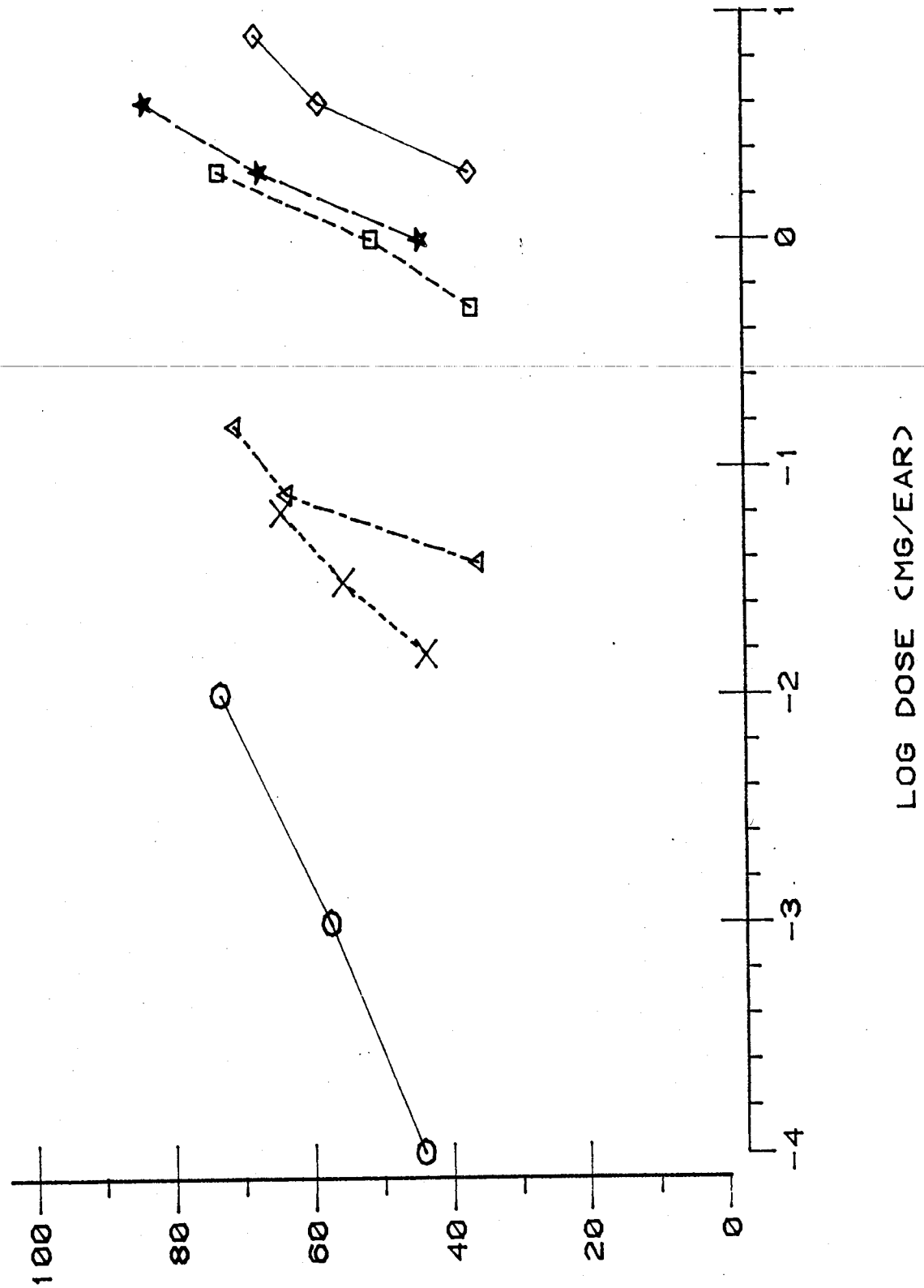
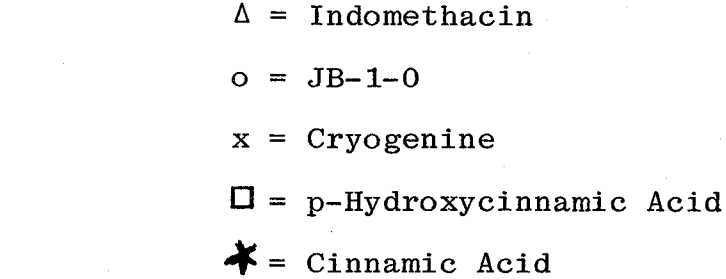

% INHIBITION EAR EDEMA
100
80
60
40
20
0
1
0
-1
-2
-3
-4
LOG DOSE (MG/EAR)

Figure 8. Log Dose-Response Curve for Cryogenine and Three Structural Components in Regard to the Suppression of Croton Oil-Induced Edema in Mice.



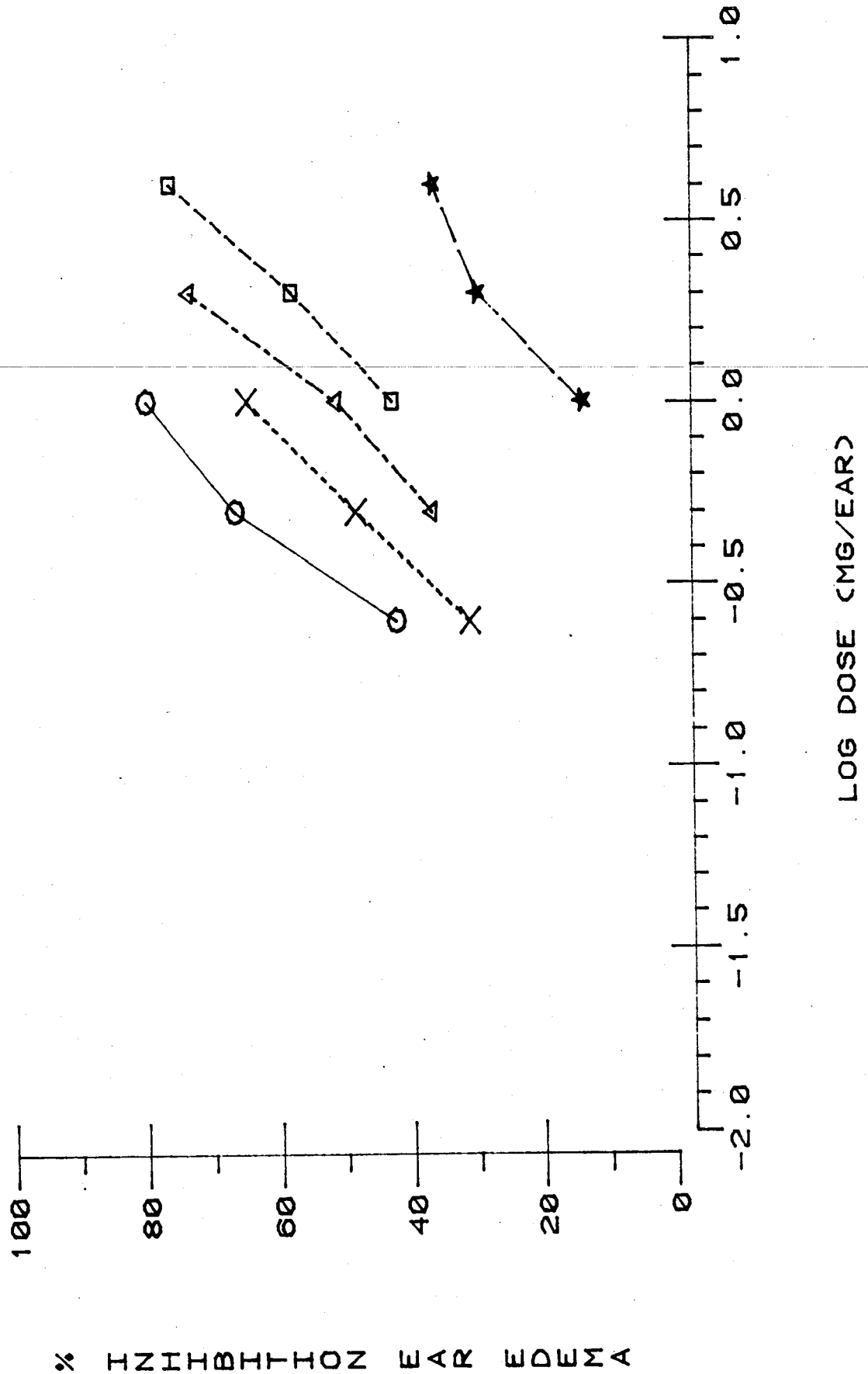

% INHIBITION EAR EDEMA
100
80
60
40
20
0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
LOG DOSE (MG/EAR)

Figure 9. Log Dose-Response Curve for Cryogenine, Lythrine, JB-1-0 and Indomethacin in Regard to the Suppression of Croton Oil-Induced Edema in Mice.

□ = Indomethacin

Δ = cryogenine

x = Lythrine

o = JB-1-0

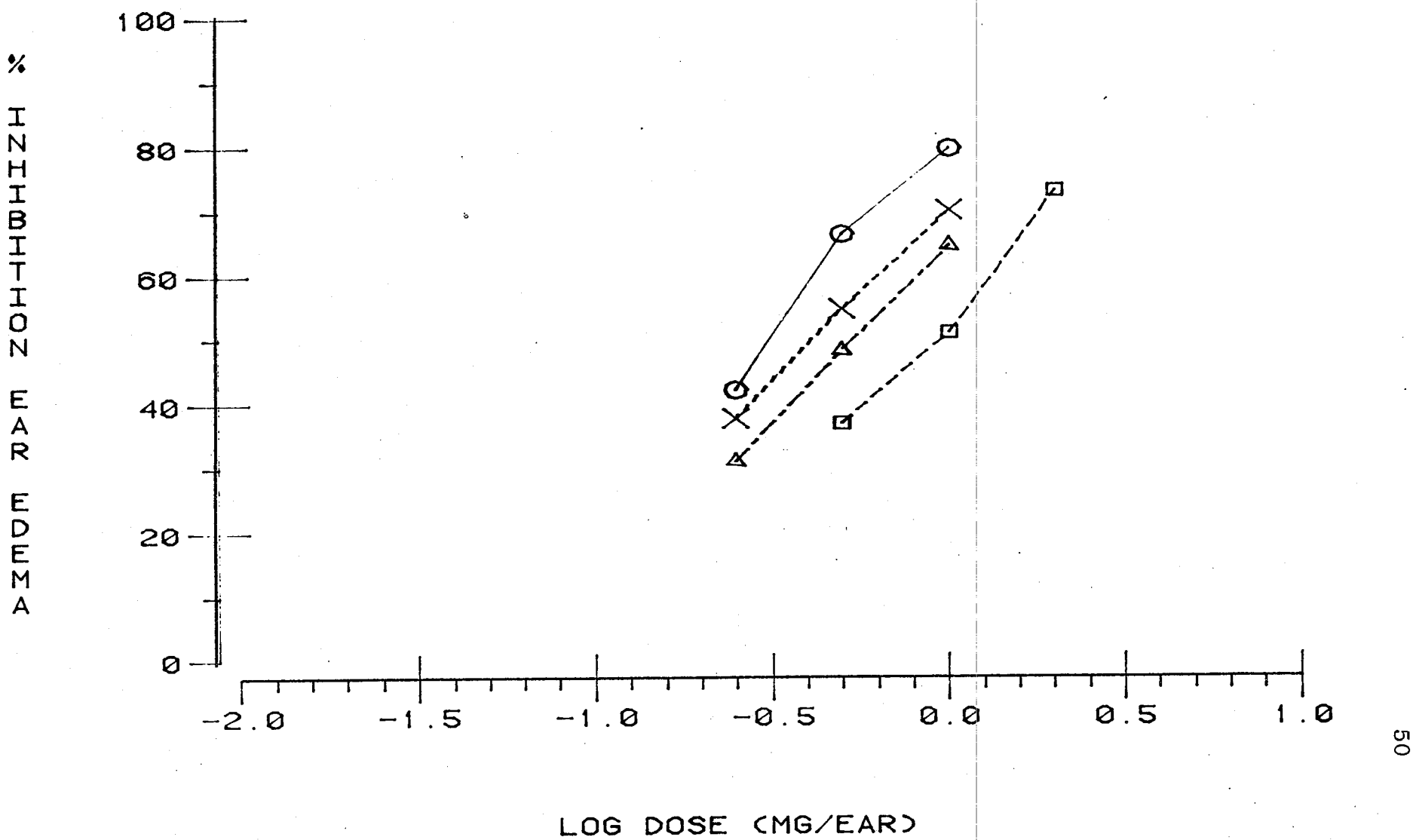

% INHIBITION EAR EDEMA
100
80
60
40
20
0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
LOG DOSE (MG/EAR)

Table VI. Comparison of Test Compounds to Indomethacin in Suppressing Croton Oil-Induced Ear Edema in Mice

Compound	Potency (95% C.L.)	Lambda	ID_{50}(mg)	ID_{50}(umoles)	Molecular Potency[b]
Indomethacin	1.0	-	9.5×10^{-1}	2.7	1.0
Dexamethasone	1780[a]	-	2.7×10^{-4}	6.9×10^{-4}	3900.0
Chlorpromazine	33.7 (20.9-54.6)	0.36	2.2×10^{-2}	7.1×10^{-2}	38.0
Diphenhydramine	14.7 (9.7-21.9)	0.30	5.3×10^{-2}	2.1×10^{-2}	12.9
JB-1-0	2.8 (1.4-2.9)	0.25	3.1×10^{-1}	8.4×10^{-1}	3.2
Lythrine	2.0 (1.4-2.9)	0.27	4.1×10^{-1}	9.4×10^{-1}	2.7
Cryogenine	1.6 (1.0-2.5)	0.33	5.5×10^{-1}	1.3	2.1
Aspirin	0.8 (0.6-1.0)	0.23	1.2	6.7	0.4
p-Hydroxy-cinnamic acid	0.6 (0.4-0.9)	0.30	1.4	8.8	0.3
Phenylbutazone	0.4 (0.3-0.6)	0.27	3.0	9.7	0.3
Cinnamic acid	0.1 (0.1-0.3)	0.38	-	-	-

[a] A lambda value and 95% C.L. for the potency cannot be calculated since the dose-response curves are not parallel.

[b] Calculated from ID_{50} estimate rather than potency.

dose response slopes were not parallel; however the mean lambda value for the remaining assays was 0.30.

It was possible to document dose-response curves that would allow valid ID_{50} estimations for all compounds except cinnamic acid for which the responses did not reach 40 percent inhibition -- a response considered as evidence of effectiveness. Kaplan, et al. (11) have shown this compound to be ineffective orally against carrageenan-induced pedal edema and adjuvant-induced polyarthritis in rats. The dose-response curve of dexamethasone was significantly flatter than those of the other compounds. This finding and its very high potency would appear to place dexamethasone in a separate pharmacologic class from the other agents tested. Considering the relatively high molecular potencies obtained for chlorpromazine and diphenhydramine, the croton oil ear edema assay is not specific for anti-inflammatory compounds. This observation is further substantiated by the ability of ethacrynic acid, a potent diuretic, to be more active than indomethacin in this assay yet devoid of any activity in the carrageenan pedal edema assay (45).

DISCUSSION

Dr. James Quick of Northeastern University and the Sheehan Institute of Research provided this laboratory with the four synthetic quinolizidine compounds illustrated in Figure 1. Since these agents had not been screened previously for their anti-inflammatory capacity, it was important to establish whether any possessed significant activity against carrageenan-induced pedal edema.

The pathway to acute carrageenan-induced inflammation has been delineated by Vinegar, *et al*. (50) and involves a twelve-step process. Edema formation is dependent on the generation of a neutrophil chemotactic factor with a subsequent neutrophil migration followed by the *in situ* generation of a vasoactive prostaglandin intermediate which increases local capillary permeability. Vane, *et al*. (51, 52) demonstrated that the common non-steroidal anti-inflammatory drugs inhibit prostaglandin synthetase and, moreover, that a correlation exists between *in vitro* synthetase inhibition

and *in vitro* anti-inflammatory activity. Subsequent investigations (48) have implicated the highly-labile prostaglandin intermediates as inflammogens since their biological activity exceeds the potency of the prostaglandins.

Of the four compounds evaluated, only JB-1-0 was effective at an oral dose of 100 mg/kg. The capacity of JB-1-0 to inhibit significantly the second stage of carrageenan-induced pedal edema is consistent with the activity of cryogenine and several clinically-effective anti-inflammatory agents; however, second-stage inhibition fails to differentiate between peripheral and centrally-mediated activity. De Cato, *et al*. (19) found cryogenine's anti-inflammatory capacity to be dependent on an intact pituitary-adrenal axis, although not affected by the central sedation induced by phenobarbital. Therefore, while the sedation observed in the JB-1-0 treated animals supports the drug's ability to penetrate the blood-brain barrier, the extent to which this involvement contributes to its anti-inflammatory activity remains to be determined. Since the molecular weights of the four synthetic quinolizidines are roughly 0.85 times that of cryogenine, effectiveness at the 100 mg/kg dose was considered to be a realistic criterion to be met if further testing was to be done. Thus, only JB-1-0 was selected for additional screening.

The croton oil-induced mouse ear edema assay is an accepted model of acute, localized edema useful in assessing

the antiphlogistic potential of topically-applied drugs. The assay allows for concurrent assessment of systemic percutaneous absorption, using thymus involution as an index of adreno-cortical activity. Janoff, et al. (53) have demonstrated marked microvascular damage with associated functional changes after a single application of phorbol myristate acetate (the active principle in croton oil) to mouse ears. This damage to the vascular endothelium is generally restricted to the venules and is accompanied by an increase in mast cell degranulation; however, these workers were unable to inhibit these acute inflammatory changes by systemic pretreatment with chlorpheniramine, 2-BOL (2-bromolysergic acid diethylamide) dibenzyline, hydrocortisone or aspirin. These data suggest that while systemic absorption of the drug substance following topical application is probable, the effect of this absorption is minor and any significant anti-inflammatory activity must be initiated by the compound at the site of application. Therefore, the inhibition of mouse ear edema induced by croton oil reflects a compound's capacity to interact with local vascular and cellular events that contribute to the acute inflammatory response.

Four of the reference compounds selected for the croton oil study are clinically useful anti-inflammatory agents. While their capacities to suppress the manifestations of acute and chronic inflammatory states run somewhat parallel,

their potencies and mechanisms of action vary with considerable complexity (54).

Dexamethasone, a synthetic steroid with high anti-inflammatory and glucocorticoid activity, is the most potent and has been shown to (i) inhibit alterations in vascular tone and capillary permeability, (ii) inhibit proliferation of capillaries and fibroblast and collagen deposition, (iii) stabilize lysosomal membranes, (iv) inhibit cellular metabolism, (v) inhibit the generation of kinins, (vi) inhibit the responses of leukocytes and macrophages, (vii) inhibit chemotaxis, (viii) alter the immune response and (ix) inhibit arachidonic acid generation from phospholipids. The croton oil-induced mouse ear edema assay displays a high sensitivity for topically-applied steroids (46). In the present study, dexamethasone was found to be 1780 times more potent than indomethacin. This value is in agreement with the work of prior investigators (46, 47) and suggests that "high potency" is a restricted domain reserved for steroids and allows for a presumptive determination regarding a test compound's corticoid-like activity.

Indomethacin, phenylbutazone and aspirin represent a class of compounds collectively referred to as non-steroidal anti-inflammatory agents (NSAI) and while each compound is currently believed to exert its anti-inflammatory activity by interfering with arachidonic acid metabolism, each possesses unique characteristic properties capable of inhibiting

several of the events that mediate acute and chronic inflammatory responses. Indomethacin has been shown to (i) inhibit 5-hydroxytryptophan decarboxylase, (ii) suppress kallikrein-induced increases in permeability, (iii) uncouple oxidative phosphorylation, (iv) inhibit mucopolysaccharide biosynthesis, (v) inhibit ameboid motility of PMN leukocytes and (vi) react with selected serum and tissue proteins. Aspirin and phenylbutazone both have the capacity to (i) uncouple oxidative phosphorylation, (ii) stabilize lysosomal membranes, (iii) inhibit synthesis of mucopolysaccharides and (iv) inhibit histamine release. However, aspirin also has been found to interact with kinin formation and suppress antibody production and antigen-antibody reactions. Furthermore, the ability of sodium salicylate to suppress clinically-established inflammation while being relatively inactive in inhibiting *in vitro* prostaglandin synthetase suggests that no single mechanism of action can adequately account for a drug's involvement with the dynamic inflammatory process.

The fact that cryogenine, lythrine and JB-1-0 all display similar dose-response profiles that closely parallel those of indomethacin, aspirin and phenylbutazone would suggest that the lythraceae alkaloids do not possess significant corticoid-like activity when applied topically. However, this would not rule out the capacity of these agents to interact with the pituitary-adrenal axis upon systemic administration.

Chlorpromazine has been shown to possess significant antiphlogistic activity in the carrageenan pedal edema assay (11). While this drug's diverse pharmacological profile suggests several potential mechanisms which may interact with the inflammatory response (55), it remains ineffective in the treatment of clinically-established inflammatory disorders. The present investigation determined chlorpromazine to be 34 times more potent than indomethacin in inhibiting the acute inflammatory response induced by croton oil. This impressive potency for a nonsteroidal agent suggests that these two agents may differ in mechanism(s) of action in this particular model of inflammation. While histamine plays a negligible role in the development of carrageenan-induced rat pedal edema (50), its involvement during the first two hours of croton oil-induced edema has been established (53). Chlorpromazine has antihistaminic capability (56), and the fact that diphenhydramine, a clinically-established antihistamine, was found to be 15 times more potent than indomethacin in the present study indicates the relatively high activity of a histamine antagonist in this particular inflammatory model. The high potency of chlorpromazine, when compared to diphenhydramine, may be due to additional localized mechanisms which also contribute to chlorpromazine's antiphlogistic capacity. The documented antihistaminic properties of cryogenine (13) may play a role in suppressing croton oil-induced mouse ear

edema; however, its capacity to inhibit carrageenan- and mycobacterium adjuvant-induced inflammations, where histamine involvement is insignificant, suggests a true anti-inflammatory potential.

The test compounds evaluated in this study (cryogenine lythrine, JB-1-0, cinnamic acid and para-hydroxycinnamic acid) were selected with the intent of determining some of the structural requirements which account for cryogenine's anti-inflammatory activity. Lythrine, the trans geometric isomer of cryogenine, was selected to assess any potential stereospecificity for molecular interactions with the hypothetical "cryogenine receptor." The potencies of these two alkaloids were found to be virtually identical; thus, unlike indomethacin (57), no apparent stereospecific conformation is preferred. This hypothesis is further supported by the activity of JB-1-0, a racemic mixture, yet relatively equipotent to cryogenine and lythrine. Since cryogenine, lythrine and JB-1-0 all have the same dimethoxy substitution pattern on the phenyl ring, the importance of this region can not be fully evaluated; however, it is noteworthy that of the four synthetic compounds screened in the carrageenan pedal edema assay, JB-1-0 was the only one which displayed significant activity. The other three compounds are substituted in the 3', 4', positions instead of the 4', 5', positions. The possibility that steric hinderance occurs when the phenyl ring is substituted in the 3' position deserves further evaluation.

Since most of the clinically-effective anti-inflammatory drugs are acidic molecules, it seems plausible to suspect the cinnamic acid moeity of cryogenine and lythrine as the active center for anti-inflammatory activity. This idea is supported by the findings of Weibelhaus, et al. (24) and two brief reports (58, 59) describing the antiphlogistic properties of cinnamic acid. Kaplan, et al. (11) found cinnamic acid to be devoid of any significant anti-inflammatory activity against carrageenan-induced rat pedal edema. While the present study found cinnamic acid to be ineffective, its para-hydroxy analog did elicit a measurable anti-inflammatory response. This analog more closely resembles the acidic function found in the lythraceae alkaloids and this is noteworthy, since Van Arman (48) contends that phenolic compounds inhibit inflammation by acting as radical scavengers. Their interaction with a highly reactive "hydroxy-radical" increases the net turnover of arachidonic acid resulting in decreased local tissue levels of the highly reactive endoperoxides and the "hydroxy-radical"--both of which have been linked to the inflammatory process. While Weibelhaus, et al. (24) imply that lythrine's para-hydroxycinnamic moeity is essential for activity, the present study found JB-1-0 to be equipotent to the two parent compounds.

Perhaps the most unique feature of the lythraceae alkaloid's anti-inflammatory capacity resides in the fact that they are nitrogenous bases. Whitehouse (60) proposed that

the acidic nature of standard non-steroidal anti-inflammatory drugs facilitates anionic binding to the lysyl E-amino groups of certain enzyme systems normally engaged in ATP biosynthesis. Scherrer (61) has described a similar receptor consisting of a large flat area, a trough to accommodate an out-of-plane group and a cationic site to accommodate acidic anions or unprotonated amines. While this model conforms to the molecular makeup of indomethacin, aspirin and phenylbutazone, it does not account for the anti-inflammatory activity of cryogenine, lythrine and JB-1-0 which would remain highly protonated at physiological conditions. Therefore, while the present study serves to help delineate the active moeity of cryogenine and lythrine and documents their topical anti-inflammatory activity (systemic and topical activity were also determined for structurally related JB-1-0), the exact mechanisms which account for the antiphlogistic potential of these compounds still remain to be determined.

Subsequent to the investigations discussed here, a series of four homologs of JB-1-0 were submitted to this laboratory for investigation as a separate study. A report of the results is included here as Appendix A. The discovery that SIR-92 exceeds the anti-inflammatory potency of JB-1-0 indicates that these compounds do indeed represent a fertile area for structure-activity research.

Recently, Lema *et al*. (62) in a preliminary study, found cryogenine to inhibit the *in vitro* synthesis of prostaglandins, displaying a dose-response profile very similar to the clinically-established anti-inflammatories aspirin and phenylbutazone. Further investigation of cryogenine's effect on prostaglandin biosynthesis coupled with an evaluation of the drug's ability to modulate cyclic nucleotide levels and alter neutrophil migration are indicated to determine whether cryogenine's unique chemical structure offers any equally unique pharmacological approach for countering the inflammatory process.

CONCLUSION

Since prehistory, innumerable plant drug products have undergone long-term "clinical trials" due to their ubiquitous employment as tribal medicinals and ritual drugs. While the reports of such use are subject to mythic distortion, they direct the pharmacologist to plants with active principles whose *a priori* safety and efficacy warrant, at least, some scientific evaluation. The wide-spread use of *Heimia salicifolia* as an ethnobotanical among the Indians of Central and South America suggests, if nothing more, a low order of toxicity in man. Since all the drugs currently available in the United States for the treatment of rheumatoid arthritis and related inflammatory conditions have undesirable side effects on chronic use, the lythraceae alkaloids may represent a potentially safe, new pharmacologic prototype with systemic and/or topical anti-inflammatory activity.

Preliminary investigation of four synthetic phenyl-

quinolizidines established that one of these compounds, 4-(2-bromo-4,5-dimethoxy phenyl) octahydro-2H-quinolizine-2-one (JB-1-0), was capable of suppressing the inflammatory response induced by the sub-plantar injection of carrageenan in rats at an oral dose which is surely effective for cryogenine.

The topical anti-inflammatory potencies of cryogenine (vertine) and lythrine (both isolated from Heimia salicifolia) and JB-1-0 were determined using the method of Van Arman (47). When applied to the ears of female Swiss-Webster mice one hour after the application of 2 percent croton oil, potencies relative to indomethacin were, respectively: 1.59 (1.01-2.50)--95% confidence limits, 2.04 (1.43-2.92), and 2.82 (2.00-4.00). Two structural components of the natural alkaloids, para-hydroxycinnamic acid and cinnamic acid, were less potent than indomethacin: 0.62 (0.41-0.93) and 0.14 (0.01-0.03). Other reference compounds included: dexamethasone, 1789 (837-3774, estimated); chlorpromazine, 33.7 (20.9-54.6); diphenhydramine, 14.7 (9.7-21.9); aspirin, 0.76 (0.55-1.04); and phenylbutazone, 0.39 (0.27-0.56). The mean lambda value for these assays was 0.30; this value excludes the dexamethasone-indomethacin assay since these two compounds have significantly different dose-response slopes.

In 1971, Chang and Malone (16) suggested that chemical modification of cryogenine could result in a new class of potent, nonsteroidal, anti-inflammatory agents. In 1973,

Weibelhaus, et al. (24) reported the alteration of lythrine's anti-inflammatory and diuretic properties by chemical reduction of the cinnamic acid moeity to form decinine. The data in the present study suggest that the reactive component for anti-inflammatory activity resides within the phenyl-quinolizidine portion of the lythraceae alkaloids rather than the cinnamic acid portion and that this activity is not stereospecific with respect to the quinolizidine ring fusion. Since bromide substitution of the phenyl ring yields a compound (JB-1-0) with significant anti-inflammatory activity in two standard assays, further substitution of the phenyl-quinolizidine ring system may well provide even more potent anti-inflammatory agents.

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APPENDIX A

DATE: April 14, 1981

TO: Dr. James Quick
SISA Institute for Research Inc.
763D Concord Avenue
Cambridge, MA 02138

RE: Report of Results of Anti-inflammatory Screening of Four Synthetic Compounds (SIR-85, SIR-86, SIR-91, SIR-92) and Suitable Controls. (Synthetic compounds Mailed by Dr. Quick to Dr. Malone, October 17, 1980)

REF: Notebook 25, pp. 229-236, 244-248.

Experimental. Because of the very limited amounts of the synthetics made available, the anti-inflammatory activity was determined using the croton oil mouse ear edema assay of Tonelli et al. (46) as modified by Van Arman (47). This assay has been shown by Byrne and Malone (63) to yield positive results for lythraceae alkaloids and a chemically related compound previously submitted to this laboratory by Dr. Quick [4-(2-bromo-4, 5-dimethoxyphenyl)-octahydro-2H-quinolizin-2-one].

Adult, healthy, female Swiss-Webster derived mice (17-20 g; Simonsen Laboratories, Gilroy CA) were housed in our facilities for at least one week prior to use and maintained on Purina Lab Chow and tap water ad libitum. One

hour prior to experimentation, the mice were randomly placed in individual cages with wide-mesh screen floors to prevent coprophagy and provided with free access to food and tap water for the remainder of the experiment. Each mouse was anesthetized with ether USP and, upon loss of righting reflex, 0.1 ml of a mixture containing 2% croton oil, 73% ether, 20% pyridine, and 5% double-distilled water was applied in four aliquots using a 25-microliter Eppendorf pipette (two applications to the anterior and two to the posterior surfaces of each animal's right ear).

One hour after the application of the croton oil-containing vehicle, the individual test compounds were dissolved or suspended at concentrations containing the specific drug dose in 0.1 ml of a vehicle consisting of 75% ether, 20% pyridine and 5% double-distilled water. The mice were again anesthetized with ether USP and 0.1 ml of drug-containing solution/suspension applied to the right ear in the manner previously described. Drug-free vehicle was applied to the right ears of the control animals.

Three hours after drug application, each mouse was sacrificed with ether USP and both the right and left ears excised at the base. A 8.0-mm circle of ear tissue was removed with a size #4 cork borer, using the outer margin of the ear as a landmark in order to avoid the extra hair and tissue associated with the base of the ear. Each section was individually placed in marked spot-test plates

to insure proper identification and then weighed to the nearest tenth of a milligram using a Sartorius laboratory balance.

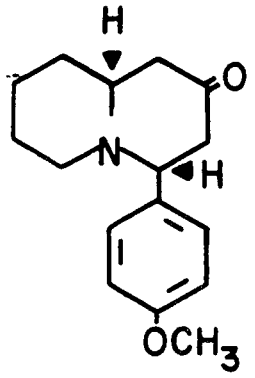
The increase in weight induced by croton oil was found by subtracting the weight of the untreated left ear from that of the corresponding right ear. Drug effects (expressed as percent inhibition) were determined by subtracting the weight increase of treated individual ears from the mean weight increase of the control group and this difference (times 100) divided by the mean weight increase of the controls.

The ID_{50} values (the dose capable of reducing croton oil-induced edema by 50 percent) and the potency estimates relative to indomethacin as a standard were calculated mathematically using regression methodology. Results are summarized in Table VII.

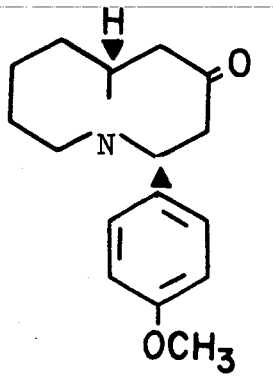
Test Compounds. The identity of the test compounds submitted are noted in Figure 10 along with their "code names" for this study. Also tested was JB-1-0, since that compound has been shown to be active using this technique (63).

Results. We originally reported that JB-1-0 was active and 2.82 times the potency of indomethacin (63). The present data indicate a potency of 2.67 times indomethacin, which figure is in very close agreement. All four of the SIR compounds were active and all somewhat more potent than

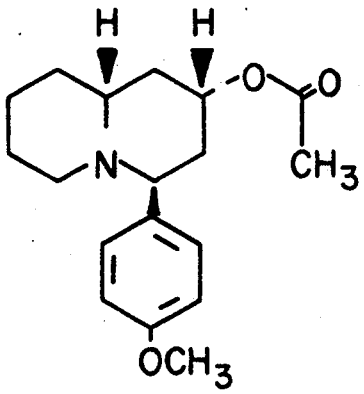
Figure 10. Structures, Code Identification, and Description of Submitted Compounds.



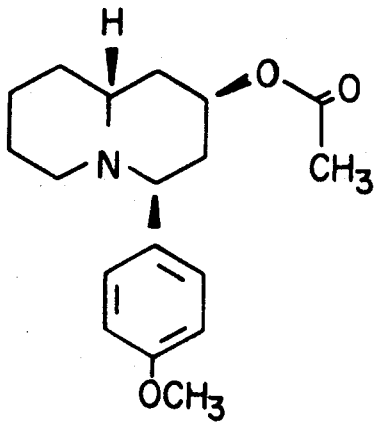
SIR-85, very light canary yellow (250 mg., m.p. 84-85, sol. dilute acid, $C_{16}H_{21}NO_2$, Batch 195.64 & 195.77)



SIR-86, creamy white (400 mg., m.p. 79-80, sol. dilute acid, $C_{16}H_{21}NO_2$, Batch 195.69 & 195.72)



SIR-91, creamy white (400 mg., m.p. 57.5-58.5, sol. dilute acid, $C_{18}H_{25}NO_3$, Batch B-208.46)



SIR-92, white fine crystals (25 mg., m.p. 130-131, sol. dilute acid, $C_{18}H_{25}NO_3$, Batch A-208.46)

Table VII. Anti-inflammatory Effects Against Croton Oil-Induced Ear Edema in Mice

Compound	Mg/Ear	Mean Percent Inhibition + 1 SEM[a]	Dose-Response Slope, b (Correlation Coefficient, r)	Dose Reducing Edema 50% ID_{50}, mg/ear	Potency
Indomethacin	0.25	39.0 ± 8.5	58.93 (0.681)[b]	0.352[b]	1.00
	0.50	63.4 ± 4.4			
	1.00	74.5 ± 2.9			
JB-1-0	0.25	62.2 ± 6.4	38.52 (0.606)[b]	0.132[b]	2.67
	0.50	69.3 ± 4.4			
	1.00	85.4 ± 2.2			
SIR-85	0.125	39.7 ± 6.1	55.84 (0.709)[b]	0.196[b]	1.80
	0.25	54.7 ± 5.8			
	0.50	73.2 ± 2.8			
SIR-86	0.25	58.2 ± 5.2	44.81 (0.525)[c]	0.164[c]	2.15
	0.50	62.8 ± 9.3			
	1.00	68.2 ± 5.1			
SIR-91	0.125	47.0 ± 7.6	41.21 (0.491)[b]	0.163[b]	2.16
	0.25	54.1 ± 8.4			
	0.50	71.8 ± 2.6			
SIR-92	0.25	76.5 ± 3.5	9.24 (0.193)[d]	$<$0.107[d]	$>$3.29[d]
	0.50	72.2 ± 3.8			
	1.00	82.1 ± 5.8			

Table VII. Continued

[a]Eight animals per test group. Inhibition expressed relative to a control (non-drug) group (N = 12) where mean edema formation was 18.59 mg (S.E.M. = 1.86).

[b]Calculated from regression equation where x = log dose per ear and y = percent inhibition (arithmetic).

[c]Only two doses (0.25 and 0.50 mg) were submitted to regression analysis, since the response of the highest dose was clearly in the asymptotic section (active response) of the dose-response curve.

[d]All three doses are in the upper asymptotic section (active response) of the dose-response curve, so estimates of ID_{50} and potency were made using graphical means. Lower doses would have been used on a subsequent day, but initial testing had consumed the entire 25 mg sample. The graphical estimate presumes that SIR-92 will have a dose-response curve parallel to that of indomethacin.

indomethacin, with SIR-85 being the least potent of the series, SIR-86 and SIR-91 being essentially equipotent, and SIR-92 being the most potent and at least 3.29 times the potency of indomethacin. The activity of SIR-92 was clearly underestimated and all doses produced what can be termed as "maximally effective" responses. Testing a range of lower doses was indicated, but the 25-mg sample had been completely consumed by the initial trials. For a nonsteroidal compound, the activity of SIR-92 is quite impressive.